**UNIVERSIDADE TÉCNICA DO ATLÂNTICO**
INSTITUTO DE ENGENHARIA E CIÊNCIAS DO MAR
**WEST AFRICAN SCIENCE SERVICE CENTRE ON CLIMATE CHANGE AND ADAPTED LAND USE**

Master Thesis

# ASSESSING BACTERIA LOAD AND ANTIBIOTIC RESISTANCE IN THE GUT OF TWO FISH SPECIES SOLD AT MINDELO FISH MARKET AND POLLUTED WATER IN CABO VERDE

***AMIE NDURE***

Master Research Program on Climate Change and Marine Sciences

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**Amie Ndure**

Master's thesis presented to obtain the master's degree in Climate Change and Marine Sciences, by the Institute of Engineering and Marine Sciences, Atlantic Technical University in the framework of the West African Science Service Centre on Climate Change and Adapted Land Use

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# Assessing Bacteria Load And Antibiotic Resistance in The Gut of Two Fish Species Sold at Mindelo Fish Market and Polluted Water in Cabo Verde

**Amie Ndure**

**Panel defense**

**President**

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**Examiner 1**

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**Examiner 2**

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São Vicente
2023


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## Dedication

This master's thesis is dedicated to my beloved father ALHAIGE NDURE, my mother AMIE KOUTA, and my husband LAMIN MAI TOURAY, who has supported me financially, emotionally, and psychologically over the pass years. To them, I owe my academic success.

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## Resumo

Este trabalho de investigação teve como foco principal, avaliar a carga bacteriana e a resistência aos antibióticos nas vísceras de duas espécies de peixe, dobrada (*Spicara melanurus*) e arenque (*Sardinella maderensis*), vendidas no mercado de peixe de Mindelo Cabo Verde e na água adjacente ao mercado. Os objetivos foram quantificar as bactérias cultiváveis nas vísceras dessas duas espécies de peixe, a sua resistência aos antibióticos normalmente utilizados, e a composição bacteriana da água do mar e do tubo imediato com resíduos de limpeza de peixe atrás do mercado de peixe do Mindelo. As amostras de peixe foram recolhidas aleatoriamente de seis vendedores diferentes do mercado de peixe, enquanto duas amostras de água foram recolhidas da água atrás do mercado, do tubo imediato que esvazia os resíduos de peixe para o corpo de água do mar, e a água do mar offshore foi usada como controlo negativo Este estudo microbiológico incluiu várias etapas, nomeadamente inoculação, isolamento, identificação usando a sequenciação, e teste de suscetibilidade a antibióticos usando a métodos dependentes da cultura bacteriana. Este estudo mostrou que a carga bacteriana foi mais elevada para *Spicara melanurus*, variando de 7×104 a 9,7×104 CFU/g, *Sardinella maderensis* apresentou uma carga de 3,3×103 a 9,3×103 CFU/g com a amostra de água do tubo mostrando uma carga mais elevada de 7,7×104, enquanto o mercado de peixe tinha 1,1×104 CFU/ml. O controlo negativo offshore não demostrou ser viável para a contagem. A microbiota intestinal das espécies de peixes e das amostras de água continha uma grande variedade de espécies bacterianas resistentes a antibióticos. Notavelmente, foi encontrada resistência à ampicilina em onze dos dezassete isolados testados. Além disso, a análise da composição bacteriana revelou a presença de bactérias potencialmente nocivas (patogénicas) na água atrás do mercado de peixe. O nosso estudo encontrou micróbios resistentes a antibióticos no intestino e no conteúdo de duas espécies vendidas na lota do Mindelo, em Cabo Verde, o que suscita preocupações quanto à potencial propagação de agentes bacterianos resistentes a antibióticos dos peixes para as pessoas.



**Palavras-chave**: *Sardinella maderensis,*Arenque, *Spicara melanurus,* dobrada, resistência a antibióticos, ambientes aquáticos**,** bactérias intestinais, Mercado de Peixe, Mindelo, São Vicente, Cabo Verde

## Abstract

This research aims to assess the bacteria load and antibiotic resistance in the guts of two species, dobrado *(Spicara melanurus),* and arenque (*Sardinella maderensis),* sold at the Mindelo Cabo Verde fish market and its adjacent water. The objectives were to quantify culturable bacteria in the guts of the two fish species. Their resistance to commonly used antibiotics, and the bacterial composition of the water with fish cleaning waste behind Mindelo fish market. Fish samples were randomly collected from six different vendors from the fish market, while water samples were collected from the water behind the market, the immediate pipe that empties the fish waste into the water body, and offshore seawater was used as a negative control. This microbiological study included several steps, namely enumeration, identification using sequencing, and antibiotic susceptibility testing using culture-dependent methods. This study showed that the bacteria load was higher for *Spicara melanurus*, ranging from $7\times10^4$ to $9.7\times10^4$ CFU/g, *Sardinella maderensis* had a load of $3.3\times10^3$ to $9.3\times10^3$ CFU/g with the water sample from the pipe showing a higher load of $7.7\times10^4$, while the fish market had $1.1\times10^4$ CFU/ml. The water samples were collected from the main waterbody adjacent to the market with the pipe that empties to it and offshore water as a negative control. The gut microbiota of the fish species and water samples contained a wide variety of antibiotic-resistant bacterial species. Remarkably, resistance to ampicillin was found in eleven out of seventeen isolates tested. Furthermore, bacterial composition analysis revealed the presence of potentially harmful(pathogenic) bacteria in the water behind the fish market. Our study found antibiotic-resistant microbes in the gut and content of two species sold at the Mindelo Fish Market in Cabo Verde, raising concerns about the potential spread of antibiotic-resistant bacterial agents from fish to people.

**Keywords:** *Sardinella maderensis*, Arenque, *Spicara melanurus*, dobrada, antibiotic resistance, aquatic environments**,** gut bacteria, Mindelo fish market, São Vicente, Cabo Verde

## Abbreviations and acronyms

| | |
|---|---|
| **AMR** | Antimicrobial-resistance |
| **ARB** | Antibiotics Resistant bacteria |
| **CFU** | Colony-forming Unit |
| **CLSI** | Clinical Laboratory Standard Institute |
| **DDT** | Dichloro-Diphenyl-Trichloroethane |
| **DNA** | Deoxyribonucleic Acid |
| **FAO** | Food and Agriculture Organization |
| **FM** | Fish Market |
| **MA** | Marine Agar |
| **MP** | Market Pipe |
| **NA** | Nutrient Agar |
| **OSCM** | Ocean Science Center Mindelo |
| **PBS** | Phosphate-Buffered Saline |
| **PCBs** | Polychlorinated Biphenyls |
| **PCR** | Polymerase Chain Reaction |
| **PG** | Picarel Gut |
| **PGC** | Picarel Gut Content |
| **RNA** | Ribonucleic Acid |
| **rRNA** | Ribosomal RNA |
| **SG** | Sardinella Gut |
| **SGC** | Sardinella Gut Content |
| **SSO** | Specific Spoilage Organisms |
| **TAE** | Tris-acetate-EDTA |
| **TE** | Tris-EDTA |
| **TVC** | Total Viable Count |
| **UV** | Ultraviolet |

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# 1. Introduction

Fish is a major source of protein for nearly 3 billion people worldwide, providing nearly 20% of the average per capita animal protein intake for more than 1.5 billion people, with percentages exceeding 50% in many countries in Africa and Asia (FAO, 2022). Moreover, essential fatty acids, including omega-3 fatty acids, which the human body cannot synthesize, are found in fish. Nutrition from fish is furthermore recognized for its low fat and cholesterol, as well as its high digestibility, making it particularly suitable for infants, children, and the elderly (Sichewo et al., 2014). However, despite its significance, fish is vulnerable to a wide diverse array of bacterial pathogens that exist with numerous variants having the potential to induce disease and are considered by some to be saprophytic in nature (Some et al., 2021).

From an infectious disease standpoint, fish and their products can carry harmful bacteria, posing health risks (Novoslavskij et al., 2016). Inadequate handling and the consumption of undercooked fish can lead to bacterial infections. Bacterial pathogens associated with fish can be categorized into two groups: indigenous types (naturally occurring in fish, such as *Vibrio* spp. and *Aeromonas* spp.) and non-indigenous types (typically not found in fish but capable of contaminating them or their environment, including *Escherichia coli, Clostridium botulinum, Shigella dysenteriae, Staphylococcus aureus, Listeria monocytogenes,* and *Salmonella spp.* (Marijani, 2022).

Investigations have been carried out to ascertain that the fish gut microbial community can be determined more by the host habitat than fish taxonomy and trophic level (Ashok Pingle & John Khandagle, 2022). Examining the taxonomic and phylogenetic similarity of fish gut bacteria from different habitats, trophic levels, and taxa revealed that environmental and ecological factors influence fish gut bacterial communities (Sullam et al., 2009). However, Kim et al., (2021) suggested that host taxonomy, or trophic level, has a stronger influence on the gut microbial community of fish.

Interest in studies investigating the prevalence of antibiotic-resistant microbes inhabiting diverse environments, especially aquatic systems, has intensified with growing understanding over antibiotic resistance and its implications for community wellness. According to Xi et al., (2009), treatment failures and higher morbidity and mortality rates.

The Mindelo fish market is a center for the trade and consumption of fish, attracting locals and tourists. As an island nation, Cabo Verde heavily depends on fish for food and revenue. Meanwhile there is a significant public health concern on the presence of bacteria resistant to antibiotics in fish. The global public health threat posed by antibiotic resistance arisen due to improper utilization and overreliance upon such medications, permitting the development and transmission of microscopic organisms immune to their effects. The spread of antibiotic-resistant seafood-borne bacteria could dangerously undermine medical interventions for human illnesses by transmitting resistant strains which may reduce treatment effectiveness (Stalder et al., 2012). Since the Mindelo fish market in Cabo Verde is a significant center for the trade and consumption of seafood, it is an ideal place to look at the existence of antibiotic-resistant bacteria in fish and surrounding ecosystems (González et al., 2020).

The Mindelo fish market, enabled measurement of the bacterial load and antibiotic resistance in the gut of two fish species Black Spot Picarel *(Spicara melanurus)* and Sardinella *(Sardinella maderensis)* sold at the fish market. The bacterial composition of water with fish cleaning byproduct behind the market was also examined. We propose to investigate the likely occurrence of antibiotic-resistant bacteria in fish and surrounding ecosystems, assess the associated dangers to public health and climate change impacts by investigating these elements. Climate change is causing global temperatures to rise, including water temperatures in oceans, rivers, and lakes. Elevated water temperatures can affect the composition and behavior of microorganisms in aquatic environments, including the gut microbiota of fish. Increased water temperatures can lead to changes in the composition of microbial communities in aquatic ecosystems. These changes can affect the gut microbiota of fish, potentially promoting the growth of antibiotic-resistant bacteria (ARB) or the transfer of resistance genes (FAO, 2022).

This document presents a study on the bacterial load and antibiotic-resistant bacteria in fish sold at the Mindelo fish market in Cabo Verde. While there have been various studies conducted on antibiotic resistance (Schar et al., 2021; Vaz-Moreira et al., 2014; Alonso et al., 2001) , this study is the first of its kind in this specific context in Mindelo, Cabo Verde since in this context of antibiotic resistance, it doesn't come across any in literature. So, this study aims to contribute to a better understanding of the prevalence and characteristics of antibiotic resistance in the neighborhood fish market. Understanding the presence and features of antibiotic- resistant bacteria in fish and their surroundings is crucial for successful public health

management and developing of antibiotic resistance mitigation measures.

The research questions of the study are as follows:

- What is the quantity of culturable bacteria in the guts of Black Spot Picarel (*Spicara melanurus)* and Sardinella (*Sardinella maderensis)* fish, and their resistance to regularly used antibiotics?
- What is the composition of culturable bacteria in the water behind the Mindelo fish market that contains fish cleaning waste?

This study has several important consequences for many parties. First, it adds to the current body of information on antibiotic resistance by investigating the existence of antibiotic-resistant microorganisms in Cabo Verdean fish and their related habitat. By conducting this study, we hope to shed light on the extent of antibiotic resistance in fish sold at the Mindelo fish market. This information will be valuable in identifying the potential risks of consuming such fish and formulating appropriate strategies to address this issue.

The study's conclusions will be useful to organizations engaged in public health, lawmaking, and fishery management. It will enable to develop effective measures to control the spread of antibiotic-resistant bacteria and protect consumers from potential health hazards. This study also contribution to the field of environmental microbiology by examining the bacterial composition of water, with fish cleaning waste. Understanding the microbial dynamics in these settings can help with the preservation and conservation of aquatic ecosystems by revealing the types and possible consequences of bacterial contamination.

### 1.1. Objectives of the study

The overarching objective of this study is to examine the existence of antibiotic-resistant bacteria in fish sold at Cabo Verde's Mindelo fish market and the related surroundings. To reach this main objective, the following specific objectives are pursued:

- To quantify the number of culturable bacteria in the guts of Black Spot Picarel *(Spicara melanurus)* and Sardinella *(Sardinella maderensis)* fish, and their resistance to regularly used antibiotics in health and aquaculture.
- To determine the composition of culturable bacteria in the water with fish cleaning waste behind Mindelo's fish market.

These objectives directed research operations and data collection, allowing for a thorough examination of the bacterial load and features of antibiotic-resistant bacteria in fish and their related habitat. The study sought to give significant insights and contribute to the knowledge of antibiotic resistance in the local fish market setting by accomplishing these objectives.

## 2. Literature review

The digestive tracts of vertebrates are colonized by complex assemblages of micro-organisms, collectively called the gut microbiota. Wang et al., (2018) revealed important contributions of gut microbiota to vertebrate health and disease, stimulating intense interest in understanding how gut microbial communities are assembled and how they impact host fitness (Wong & Rawls, 2012).

Due to its substantial protein content, rich abundance of unsaturated fatty acids, and minimal carbohydrate content, fish serves as a crucial nutrient source for humans. Consequently, the consumption of fish is recommended to mitigate the risk of lifestyle diseases associated with the intake of red meat (Shikongo-Nambabi et al., 2011).

Furthermore, a study by (Beyari et al., (2021) showed that the composition of different parts of the fish varies greatly. Fish protein content averages around 19%, it can range from 6% to 28%. The cholesterol oil content may vary between 0.2% and 64%, the ash content between 0.4% and 1.5%, and the moisture content between 28% and 90%. The majority of these species exhibit a low oil content and a high protein content. The cause of variation in the proximate composition of fish, which is frequently attributed to factors such as geographical area or season, is primarily related to the feed ingested, the fishes' mobility and metabolic rate. The mineral and fat-soluble vitamin content of sea foods is slightly higher than that of terrestrial animals. The species, fat content presence and type of non-protein nitrogenous compounds all affect the flavor of sea foods (Beyari et al., 2021; Venugopal, 1996).

Due to the nature of the muscle tissues, fish is a very perishable food. Fish tissue deteriorates more quickly than the muscles of mammals (Furnesvik et al., 2022; Venugopal, 1996).. The high water and free amino acid content, and the lower content of connective tissue as compared to other flesh foods lead to the more rapid spoilage of fish (Masniyom, 2011). Fish could be contaminated by microorganisms and/or chemical substances, such as those containing heavy metals, organochlorine pollutants like Polychlorinated Biphenyls (PCBs), Dichloro-Diphenyl-Trichloroethane (DDT), dieldrin, chlordane, and dioxins (Beyari et al., 2021;Acharjee et al., 2014) and accidental petroleum spills and radioactive materials of anthropogenic origin (Hubanova et al., 2019; Law et al, 1999).

Around the world, seafood-borne diseases are a major public health concern. Seafood consumption has increased in recent decades, globally, per capita. Both imports of seafood and domestic aquaculture farming have increased. Additionally, the consumption of contaminated

seafood has been linked to a number of recent outbreaks of human gastroenteritis (Elbashir et al., 2018). The examination of seafood-borne illnesses caused by norovirus, *Vibrio*, and other bacteria and viruses had facilitated a thorough understanding of the pathogenicity and virulence properties of the etiologic agents investigated pathogens associated with seafood and resulting outbreaks in the United States and other countries, as well as the presence of antimicrobial resistance in the pathogens examined. The antimicrobial overuse, misuse, and sub-therapeutic application in humans and animals has broaden the spectrum of such resistance (Elbashir et al., 2018).

The gut microbiota exerts a profound influence on both overall health and immunity in fish, as supported by recent research (Okechalu et al., 2023; Butt et al, 2019; Zeng et al., 2020). In various fish species, dominant phyla, including Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria, and Fusobacteria, have been identified within the gut microbiota. However, it is essential to note that the specific composition of the gut microbiota can vary significantly between different fish species and even among individual fish (Butt et al, 2019).

These studies collectively establish that the gut microbiota in fish plays a pivotal role in regulating a wide array of host physiological processes. This includes critical functions such as feeding, digestion, metabolism, stress management, reproductive health, development, and immune responses. Numerous factors, both external and internal, contribute to the composition and dynamics of the gut microbiota in fish. These factors include species-specific differences, developmental stages, dietary composition, habitat conditions, environmental factors like water temperature and salinity, as well as the conditions under which fish are raised (Sheng et al, 2021). Ultimately, the well-being and immune resilience of fish are intricately linked to the health of their gut microbiota (Tseng et al., 2018).

Microorganisms in the marine environment have harmful and beneficial functions. They manifest biogeochemical features that are critical process in marine environments, the majority of microbial contaminants consist of pathogenic bacteria, viruses, or eukaryotic parasites (Hewson et al., 2007). Direct contact with a contaminated water environment, as well as the consumption of bacteria from sediments or contaminated feed, are all contributors to the presence of various bacterial species in fish, including human pathogenic bacteria, as such bacteria found in fish serve as an indicator of aquatic environments (Novoslavskij et al., 2016).

Microbial contamination after harvesting can have an impact on the nutritional qualities and shelf life of fish. Balogun et al., (2019), looked into the microbiological analysis of fish as well as the antibiotic sensitivity pattern of isolated bacteria from some selected fish sold in Ibadan's

Bodija Market. With different zones of inhibition, *Escherichia coli* showed the greatest susceptibility to all antibiotics. Most strains of *Salmonella* were susceptible to septrin and the smallest inhibition zone to pefloxacin. *Shigella spp*. had the lowest zone of inhibition to gentamicin, rocephin, septrin, and erythromycin when compared to other bacterial isolates. The largest gentamicin inhibition zone was found in *Serratia spp*. Balogun et al., (2019). Every isolated bacterium displayed heightened resistant antibiotics, contributing to the emergence of antibiotic-resistant strains.

The fish gut serves as a breeding ground for antibiotic-resistant bacteria, posing a significant (Larsson et al., 2018; *WHO*, 2015). The proliferation and dissemination of antibiotic-resistance genes within the fish gut have been exacerbated by the inappropriate and excessive utilization of antibiotics in aquaculture practices. Antibiotic-resistant bacteria are causing an increase in the number of infections worldwide (Ben et al., 2019). Fish from multi-source waters may harbor multidrug-resistant bacteria, which can be transmitted to humans through consumption or contact with contaminated fish. Gufe et al., (2019), did a cross-sectional study and found out that *Pseudomonas*, *Citrobacter*, *Klebsiella*, *Enterobacter*, and *Proteus mirabilis* are multiple antibiotic resistance (MAR) indexes of 0.2, while the other isolated bacteria have MAR indexes ranging from 0.3 to 0.7. MAR indexes greater than 0.2 indicated that the bacteria isolates came from a high-risk source where antibiotics were frequently used, possibly sewage effluent. (Tyagi et al., 2019), found, the presence of pathogenic bacteria and antibiotics resistant genes (ARGs) on plasmid sequences suggesting that the potential risk of horizontal gene transfer in the confined gut environment, could allow the gut microbiota of fish to acquire antibiotic resistance genes, further exacerbating the issue of antibiotic resistance.

Ryu et al., (2012), looked into antimicrobial resistance and characterized the genes involved in *Escherichia coli* isolated from commercial fish and seafood. They gathered fish and seafood samples (n=2663) from wholesale and retail markets in Seoul, Korea, between 2005 and 2008. In the samples, 179 *E. coli* isolates, amounting to 6.7%, underwent antimicrobial resistance testing. Out of the 179 isolates, seventy were found to be resistant to one or more drugs, and these were subsequently subjected to testing for the presence of three categories of antimicrobial resistance genes, namely tetracycline, aminoglycosides, and beta-lactams. Integrons of classes 1, 2, and 3, ampliconsequencing was used to characterize gene cassettes from classes 1 and 2 integrons, and their findings imply that commercial fish and seafood may serve as a reservoir for multi-resistant bacteria, facilitating the spread of the resistance gene (Ryu et al., 2012).

Antibiotic resistance is spread throughout the fish gut by several factors. These factors encompass environmental pollution, antibiotic utilization in aquaculture, interactions between fish and their gut microbiota, and the existence of mobile genetic elements. By directly applying selective pressure to gut bacteria, antibiotics used in aquaculture can promote the survival and spread of resistant strains. It is possible for antibiotic resistance to emerge and persist. Research endeavors concerning antibiotic resistance and the detection of resistance genes in bacteria extracted from water, sediment, and fish within trout farms have guided researchers to the deduction that the aquatic environment could exert a noteworthy influence on the development of antibiotic resistance and the dissemination of resistance genes among bacterial populations (Capkin et al., 2015). As a result of environmental contamination, like the release of antibiotic residues into aquatic ecosystems (Sheng et al, 2021). Sayah et al., (2005), (investigated 1,286 *Escherichia coli* strains isolated from human septum, wildlife, domestic animals, farm environments, and surface water for patterns of antimicrobial resistance in the Red Cedar watershed in Michigan. *E. coli* was isolated and identified using enrichment media, selective media, and biochemical tests. The research found antimicrobial resistance in isolates from livestock, companion animals, human septage, wildlife, and water on the surface in general, she found that *E. coli* isolates from domestic species were more resistant to antimicrobial agents than isolates from human septage, wildlife, and surface water (Sayah et al., 2005), multidrug resistance was found in a variety of sources, with swine fecal samples having the highest levels of multidrug-resistant *E. coli*. The water sample isolates were only resistant to cephalothin suggests that resistance patterns in farm environment samples may be more representative of the risk of antimicrobial agent-resistant bacteria contaminating surface waters (Sayah et al., 2005).

While we have gained substantial insights into bacterial load and antibiotic resistance within fish guts, numerous knowledge gaps remain, offering promising avenues for future research. More research is needed with a larger variety of fish species and sample sites to necessary provide a representative understanding of the bacterial load and antibiotic resistance profiles in fish sold at the Mindelo fish market and water body, as various species may display differences in bacterial load and antibiotic resistance profiles. To completely understand how antibiotic resistance appears and spreads in fish gut microbiota too needed to be look into.

A thorough analysis of the bacterial load and antibiotic resistance in the guts of fish sold at the Mindelo fish market in connection to the water body is necessary to comprehend the dynamics of antibiotic resistance transmission in aquatic ecosystems. This analysis lays the groundwork

for further investigation while highlighting the importance of continuous management strategies to lessen the risks brought on by fish gut microbiome antibiotic resistance.

Despite current studies on bacterial load and antibiotic resistance in fish gut microbiota, the literature has gaps and disputes. The absence of established methods for measuring bacterial burden and antibiotic resistance in fish is one of the drawbacks. Due to variations in sample methodologies, culture media, and laboratory procedures, it becomes challenging to make direct comparisons and draw general conclusions across different research studies. Furthermore, research concentrating on more species of fish widely sold at fish markets is needed.

# 3. Materials and Methods

## 3.1 Study site

The study was conducted at the Mindelo Fish Market in São Vicente, Cabo Verde (Figure 1). The Mindelo Fish Market (Figure 1B), located in the bay of Porto Grande in São Vicente, is one of the island's most iconic locations. In addition to providing a source of income for the fishermen and fishmongers who congregate at the fish market, people visit the place almost daily in search of fish, one of the essential products in the Cabo Verdean diet. The market is in a strategic location of the bay. Because of this, other activities that take place there, it is increasingly becoming a tourist attraction for visitors to the island. As per the United Nations classification, Cabo Verde is a medium human development country, boasting a total population of 491,683 individuals. Among them, 76,107 of the population reside on the island of São Vicente(Garcia Rodrigues & Villasante, 2016). São Vicente's fishing endeavors, illustrated in Figure 1B, predominantly revolve around small-scale fisheries, targeting diverse species that encompass low trophic level demersal fish and larger, higher trophic level pelagic fish. Among these species, the Black Spot Picarel *(Spicara melanurus)* and Sardinella (*Sardinella maderensis)* are affordable and widely consumed by the local population. The Mindelo municipal fish market serves as the main market place on the island for fresh fish, where fish cleaning is also done. The market is located close to the harbor where the fish is landed, buying transactions take place before the vendors takes it to market to retail it.

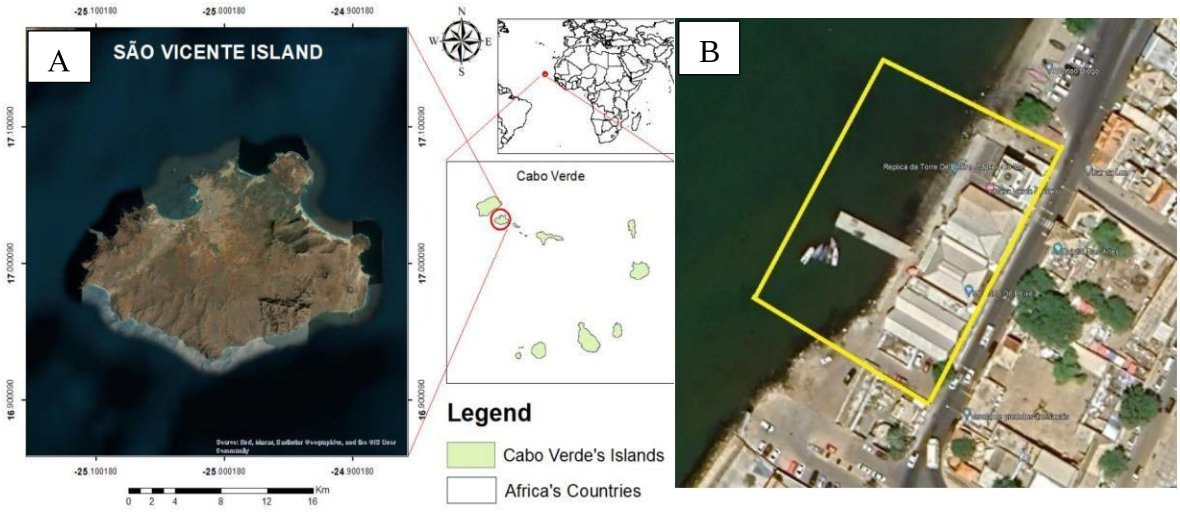


**Figure 1**: Study Site. A) Map of Africa showing the Location of Cabo Verde and Mindelo in Cabo Verde, Source: Goggle Maps. B). The Mindelo Fish Market Source: "Resilient Tourism and Blue Economy Development in Cabo Verde" Project.

### 3.2 Data collection

Two different fish species were collected from the Mindelo Fish Market: Sardinella *(S. maderensis)* (Figure 2A) and Black Scad Picarel *(S. melanurus)* (Figure 2B), and water samples were collected from the harbor and the pipe behind the market and offshore as a control, 10 ml of the water sample was put in tubes with 2 ml glycerol. A total of 6 fish samples, three for each species, were collected. Yes, the idea of the research is to get base line information on what is found in the fish and water. My opinion is the research is an eye opener to those in charge. Fish samples were randomly collected from six different vendors at the Mindelo Fish Market and transported in a cooler box with ice at a temperature of 4˚C to the Ocean Science Center Mindelo (OSCM) laboratory. Extraction of the gut were conducted after the fish skin was clean with 70% ethanol to reduce any accidental organism on the surface. The bacterial isolates from each specimen were obtained aseptically from the gut with sterile dissecting instruments and immediately frozen at -80˚C freezer with the water samples. The water and gut samples were shipped at -80˚C to Germany for testing and analysis.


**Figure 2**: The two different fish species samples. A) Sardinella (*Sardinella maderensis*). B) Black Spot Picarel (*Spicara melanurus*).

### 3.3 Sample preparation

The sample isolates from each specimen were obtained aseptically from the gut with sterile dissecting instruments (Andrews & Hammack, 2003). To determine the cultivable bacteria associated with intestinal epithelium and content, the method described by Ringø et al., (2016) was applied. The entire gut from the stomach to the anus was removed, and the contents were stripped out with sterile forceps. Gut and content were transferred to microcentrifuge tubes and weighed. Net wet weights were reported for calculation of bacterial load per mass of gut and gut contents. Then, the gut and contents were smashed in a tissue grinder and homogenized

with 200 µl of PBS. Sample weight varied from 0.5 g (wet weight) to a maximum weight of 0.8 g. Obtained gut and gut content were divided into two different tubes and were used for inoculation and extraction.

For intestinal epithelium bacteria, the gut content was emptied and rinsed with 1x PBS to clean the content further (Hasegawa et al., 2017). After the samples were weighed, the samples were transferred to appropriate tubes, smashed in a tissue grinder, and homogenized with 200 µl of PBS for both gut and content.

### 3.3.1 Sample inoculation

For sample inoculation, it was made a serial dilution of $10^{-1}$, $10^{-2}$ and $10^{-3}$ by diluting 100 µl from the supernatant to 900 µl PBS. 100 µl of each dilution was used as an inoculant and spread in the petri dish with three replicates for the fish sample. A similar method was used for the water samples. The water samples were collected in sterilized bottles. Three serial dilutions were made by adding 1 ml from the supernatant to 9 ml peptone water of 0.18% for the harbor and pipe but the offshore B was not serially diluted. 100 µl of each dilution was poured into sterilized nutrient agar plates and incubated at room temperature (20˚C to 25˚C) for 24 hours. The procedure was done in a working hood (Figure 3A), which is a sterilized environment with UV light and air aeration for ventilation to avoid any possible contamination. The incubator (Figure 3B) was used to keep the samples at optimum temperature (21˚C) that promote growth of bacteria on inoculated plates. The protocol is detailed in Appendix 9.1.

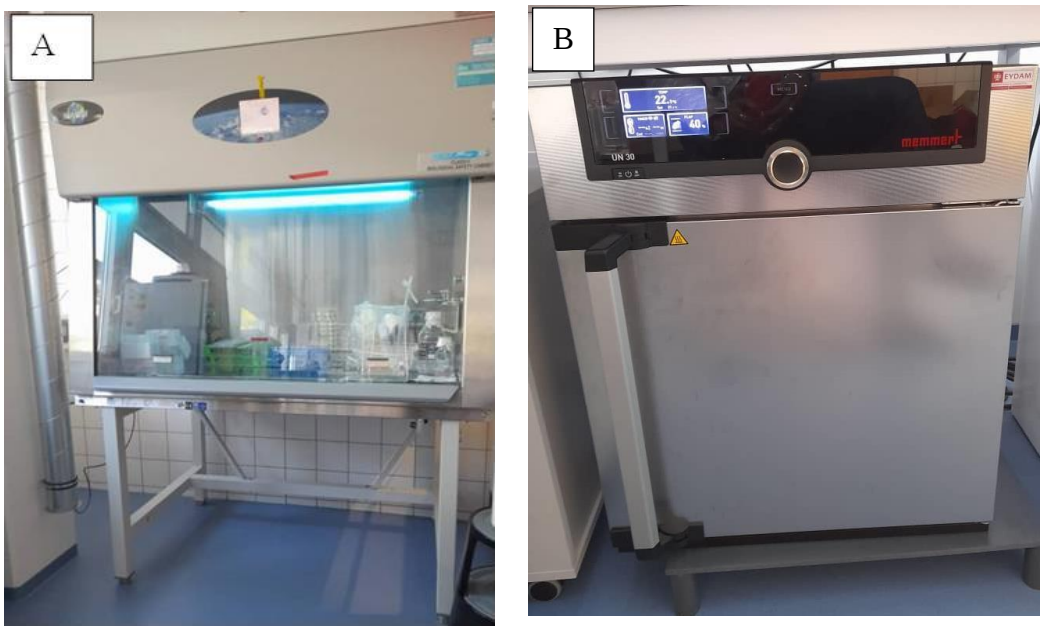


**Figure 3**: Working environment for inoculation and incubation. A) Working hood and B) Incubator.

### 3.3.2 Media cultivation procedures

Nutrient Agar (NA) and Marine Agar (MA) were used as cultivation media. An aliquot of 100 µl of each desired sample solution was spread on each culture medium and incubated at room temperature (approximately 20˚C to 25˚C) for 24 hours to 37 hours. Inoculation of bacterial load in samples was done by spread plate method. The total colony forming units (CFU) per gram of sample was calculated using standard methods (SLABYJ et al., 1981). The mean colony forming unit per gram (CFU g-1) denoted by (x) was calculated as $\Sigma f\chi/\Sigma f$, where $\Sigma f\chi$ is the sum of the products of number of colonies and the colony forming unit per gram, while $\Sigma f$ is the summation of the number of colonies. Water samples were calculated as CFU/ml = (No. of colonies x Total dilution factor) / Volume of culture plated in ml (SLABYJ et al., 1981).

### 3.3.3 Isolation and identification of species

Ten distinct colonies based on morphology were picked randomly from positive plates (Figure 4A) in both water and fish using a sterile plastic loop and sub-cultured (Figure 4B), then transferred onto a freshly prepared nutrient agar medium contained in sterile plates, incubated at room temperature (approximately 20˚C to 25˚C) for 24 hours and part of the same colony immersed in 50 µl molecular graded water for DNA extraction and amplification.

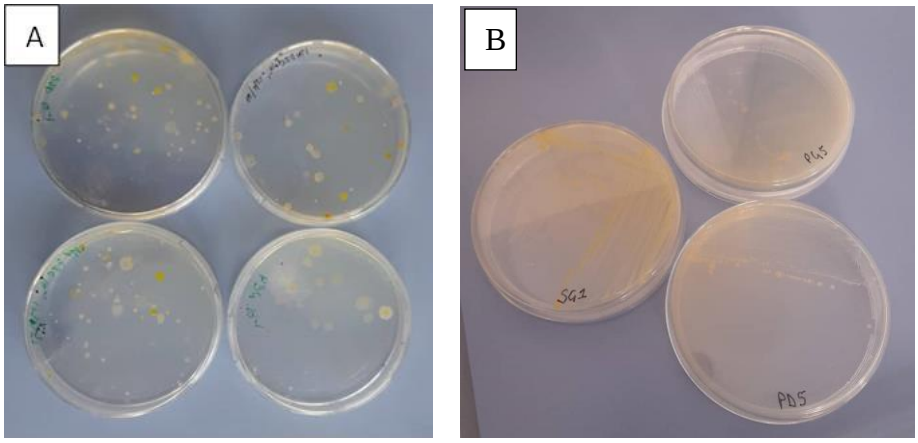


**Figure 4**: Inoculated plates. A) Positive plates. B) Subculture plates.

The samples for molecular microbiology were kept in -20˚C freezers until use. Several colonies were subjected to PCR amplification using 16S rRNA forward (27F: AGAGTTTGATCCTGGCTCAG) and reverse (1392-R: ACGGGCGGTGTGTGTAC) primers. The master mix, comprised of 2 µl dream buffer (10X), 0.4 µl dNTPs (10mM), 0.8 µl 27F (10µM), 0.8 µl 1392-R (10µM), 0.5 µl Dream Taq and 14.5 µl molecular grade water for each sample. The total amount of these mixed reagents (19 µl) was added to cryotubes with 1 µl of the colony that had been previously diluted in 50 µl molecular water. PCR amplification

of 35 cycles was carried out in a Thermocycler (Figure 5A). See whole protocol in Appendix 9.2. The cryotubes were stored in a freezer at -20˚C. The same analyses were carried out on water samples collected from the harbor and pipe at the market.

For gel electrophoresis, agarose gel was prepared from 150 ml Tris-acetate-EDTA (TAE) buffer and 2.25 g agarose mixed well and melted in the microwave. Six drops of ethidium bromide were then added, and the gel was cast on a tray with 32 wells solidified. After putting the tray in the DNA Electrophoresis System **(**Figure 5B). 5 µl was taken from each PCR reaction mixed with 1 µl of dye loaded in the wells starting from the second well and ending with negative control. 1 µl ladder, 1 µl dye and 8 µl molecular graded water were mixed and loaded in the first well as a ladder to estimate the molecular size of the PCR products. Electrophoresis power was set at 100 V for 30 minutes to determine the band sizes of the PCR products based on the distance travelled Ringø et al., (2016), see protocol in Appendix 9.3. This was repeated for the rest of the samples.

The results were visualized using the UV transilluminator gel electrophoresis (Biostep camera) **(**Figure 5C) and read with the laptop**.** The five strongest bands from each sample were selected and sent for sequencing at (Eurofins). The sequence of 16S rRNA gene was determined by Sanger sequencing Protocol (Appendix 9.4), using Applied Biosystems 3730xl DNA Analyzer. The 27 F and 1392 R primers were used for setting up PCR reactions using the Big Dye Terminator from ABI. Sequences from forward and reverse primers were aligned using Vector NTI software (Invitrogen). Classification of sequences was done using BLAST and comparison of sequences currently available in the national center for biotechnology information (NCBI) database (Zhang et al., 2000).

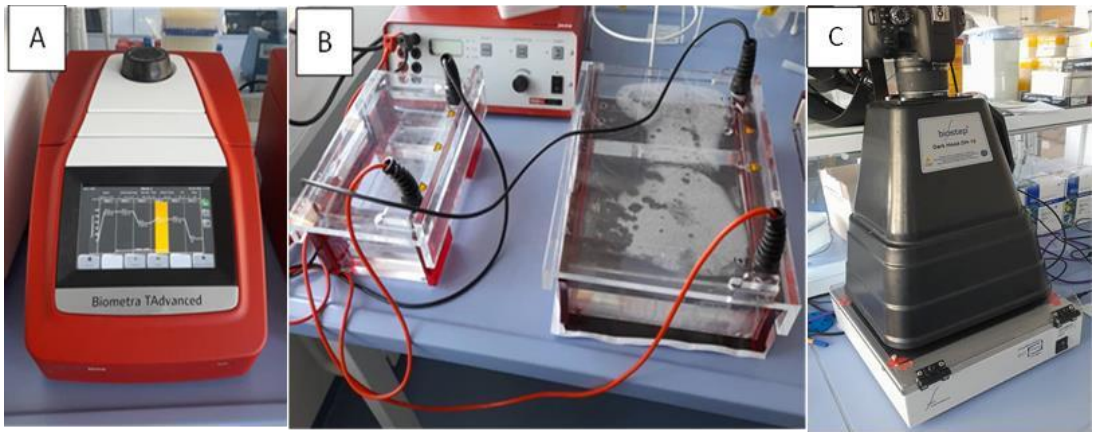


**Figure 5**: Equipment's used for DNA amplification. A) Thermocycler (PCR Machine or DNA Amplifier) Biometra PCR Systems. B) DNA Electrophoresis System. C) UV transilluminator for gel electrophoresis (Prime Gel Documentation).

### 3.3.4 Antimicrobial susceptibility test

The antimicrobial susceptibility test was done on seventeen samples: six from water samples, six from sardinella (*S. maderensis)* gut and content, and five from the picarel (*S. melanurus)* gut and content. These five were isolates from identification that are unique from the gut and content of (*S. melanurus*), the other were the same isolate names that's why we choose five. These isolates were subculture from the pure plate. Overnight cultured isolate was used (Figure 6A) for the test. The susceptibility test was performed using the disc diffusion method (Hudzicki, 2012). The isolates were tested against four antibiotics, namely, ampicillin (10 µg); erythromycin (10 µg); streptomycin (10 µg); and chloramphenicol (30 µg). Ampicillin, erythromycin, streptomycin are commonly used in clinical setting in treating bacterial infection and chloramphenicol is used in aquaculture.

For antimicrobial susceptibility assays, a number of bacterial colonies was used to prepare suspensions corresponding to 0.5 McFarland standards ($1.5 \times 10^8$ CFU/ml) using normal saline, then bacteria isolates were spread onto the Mueller-Hinton agar using a sterile loop and allowed to dry for 2 to 5 minutes. Antibiotic discs were immediately placed on the surface of the agar plate using forceps and incubated at room temperature (approximately 20°C to 25°C) for 24 to 37 hours. Zones of inhibition (Figure 6B) were measured by means of a simple ruler, and the diameter was recorded in milli-meters (mm). The antibiotic disk size being 6 mm in diameter, the smallest zone of inhibition measurement diameter is 6 mm; that is, measurements of 6 mm can be interpreted as resistant. Conversely, the 6 mm diameter could be subtracted to determine the zone of inhibition minus the disk (Hudzicki, 2012). Isolates were defined as susceptible, intermediate, or resistant in accordance with the CLSI M100-Ed33 Enterobacteriaceae breakpoints (Ruzauskas et al., 2021).

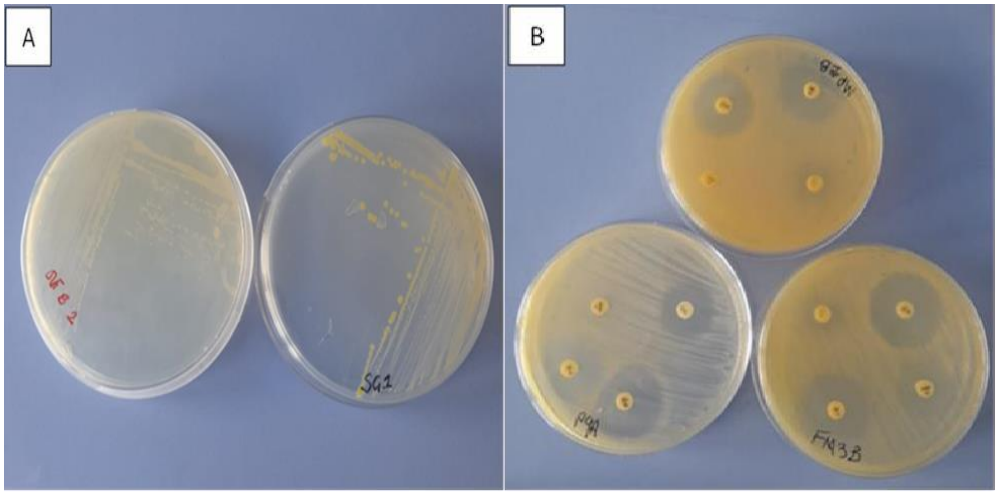


**Figure 6**: Plates used for antibiotic tests. A). Overnight cultured plates. B). Susceptibility test plates.

# 3. Results

## 4.1 Total count of viable colonies (TVC)

We manually enumerated colonies on plates that displayed 25 to 250 colonies each. The samples from picarel (*S. melanurus)* exhibited the highest bacterial load, with viable counts ranging from $7\times10^4$, $8.6\times10^4$, $8.7\times10^3$, $9.4\times10^3$, to $9.7\times10^4$ CFU/g. In comparison, the samples from sardinella (*S. maderensis)* showed a noteworthy disparity from the Picarel counts: $3.4\times10^3$, $3.8\times10^3$, $4.2\times10^3$, $4.3\times10^3$, $5.2\times10^3$, $5.8\times10^3$, $8.1\times10^3$, and $9.2\times10^3$ CFU/ml.

Furthermore, the results revealed a vast difference between the two water samples, with higher bacterial load found in the pipe samples, quantified at $7.7\times10^4$ CFU/ml, compared to the fish market samples, which had $1.1\times10^4$ CFU/ml. This difference between the two samples was considerable. Notably, the offshore sample used as a control was non-viable for enumeration. It was not viable for counting but we go ahead with the few that grow to do the isolation and identification that why we are able to know that there is *Psedoalteromonas* in it. These outcomes underscored a marked variation among the different samples, as depicted in (Figure 7).

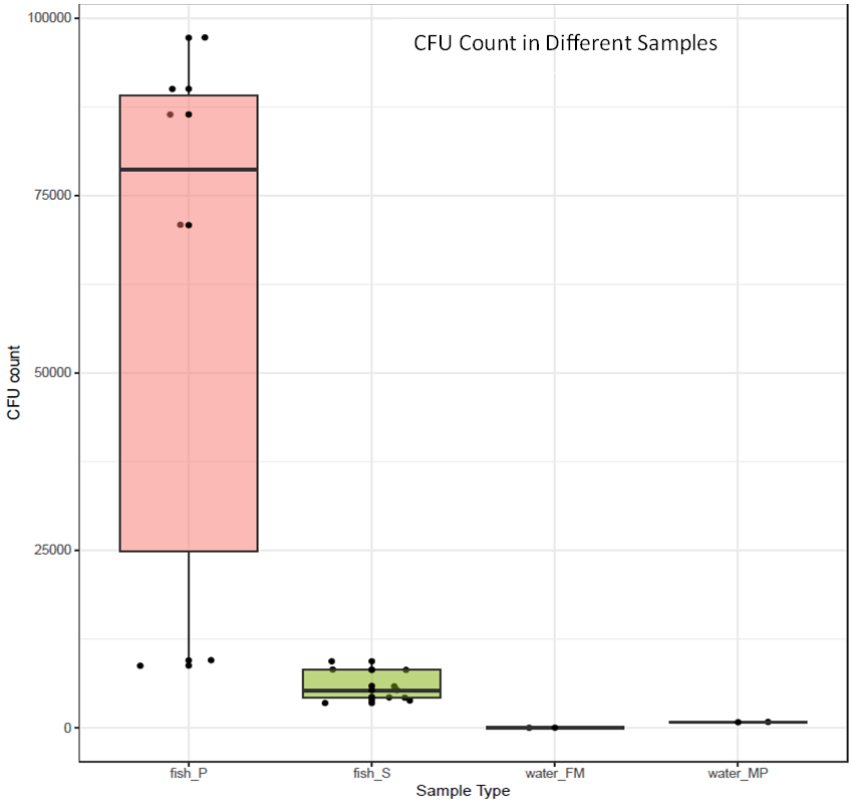


**Figure 7**: Bacterial density in different samples from positive plates fish_P is picarel (*S. melanurus)* samples, fish_S is sardinella (*S. maderensis)* samples per gram, water_FM is water samples from the market and water_MP is water samples from the pipe per ml.

### 4.2 Identification of isolates

Five strongest bands from the gel read of each water sample fish market (FM), market pipe (MP) and three from offshore (OUT B) were sent for sequencing. The fish samples ten strongest bands were also selected for each species, five for sardinella (*S. maderensis*) gut (SG*)* and five for sardinella (*S. maderensis*) gut content (SGC) with five for picarel (*S. melanurus*) gut (PG) and fivefor picarel (*S. melanurus)* gut content (PGC). A total of 29 different bacteria isolates belonging to 12 genera from both water and fish samples were found. One isolate from the sardinella gut content was not successfully sequenced and is indicated as unknown. One initial isolate *Moraxella spp.* (isolated from the Market Pipe) was considered "Biosafety 2" which means it can cause disease in humans, hence it was discarded to preserve the biosafety of the lab. *Pseudoalteromonas* from offshore (OUT B) all have the same GenBank Accession number, and other isolates have the same name but different accession numbers (Table 1).

**Table 1**: Summary table of all bacteria isolates found from the sequencing results with GenBank Accession numbers.

| Samples | Bacteria Isolates | GenBank Accession number |
|---|---|---|
| FM | *Pseudomonas sp.* | MT555352 |
| FM | *Brochothrix thermosphacta* | MN062089 |
| FM | *Pseudomonas fragi* | MH463554 |
| FM | *Pseudomonas fragi* | KC854411 |
| FM | *Brochothrix thermosphacta* | MN062089 |
| MP | *Pseudomonas weihenstephanensis* | MN062086 |
| MP | *Pseudomonas fragi* | CP104861 |
| MP | *Pseudomonas fragi* | KP745584 |
| OUT B | *Pseudoalteromonas* | KR012161 |
| OUT B | *Pseudoalteromonas* | MG799459 |
| OUT B | *Pseudoalteromonas* | MN126741 |
| SGC | *Psychrobacter glacincola* | AB334769 |
| SGC | *Lactococcus piscium* | OX460936 |
| SGC | *Staphylococcus saprophyticus* | CP054831 |
| SGC | *Shewanella beltica* | LR134321 |
| SG | *Glutamicibacter bergerei* | MK424283 |

| | | |
|---|---|---|
| SG | *Psychrobacter sp.* | EU345114 |
| SG | *Psychrobacter cibarius* | OP716171 |
| SG | *Glutamicibacter bergerei* | MK424283 |
| SG | *Halomonas* | MN043765 |
| PGC | *Pseudoalterononas* | FJ457147 |
| PGC | *Pseudoalterononas* | FJ457148 |
| PGC | *Pseudoalterononas* | FJ457149 |
| PGC | *Turicibacter sp.* | MK287738 |
| PGC | *Shewanella sp.* | EU807746 |
| PG | *Shewanella sp.* | CP113803 |
| PG | *Shewanella putrefacient* | MK967210 |
| PG | *Photobacterium leiognathi* | AY292944 |
| PG | *Shewanella beltica* | AB205580 |
| PG | *Shewanella beltica* | HM584022 |

### 4.3. Diversity of bacteria isolates in water samples

From the subset of the bacteria isolated, the morphologically diverse colonies had three distinct genera. The Market exhibited the highest diversity, with four distinct bacterial isolates. This was followed by the Pipe, which had two isolates, and the offshore location, which had only one isolate. Interestingly, *Pseudomonas fragi* was identified in both the Market and Pipe samples, as illustrated in (Figure 8).

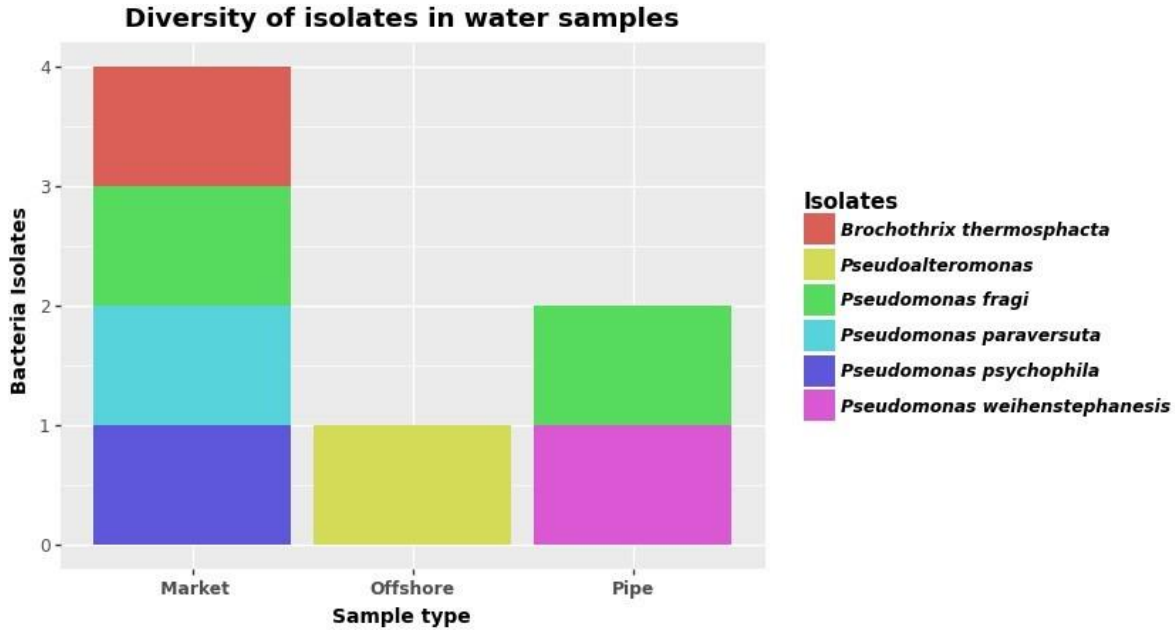


**Figure 8:** Different isolates found in the water samples. Each color represents an isolate.

### 4.4. Diversity of bacteria isolates in *sardinella* samples.

The diversity of bacterial isolates between the gut and gut content of sardinella (*S. maderensis*) reveals nine distinct isolates belonging to eight genera. Among these, five isolates were present in the gut content, and four isolates were found in the gut, as illustrated in (Figure 9).

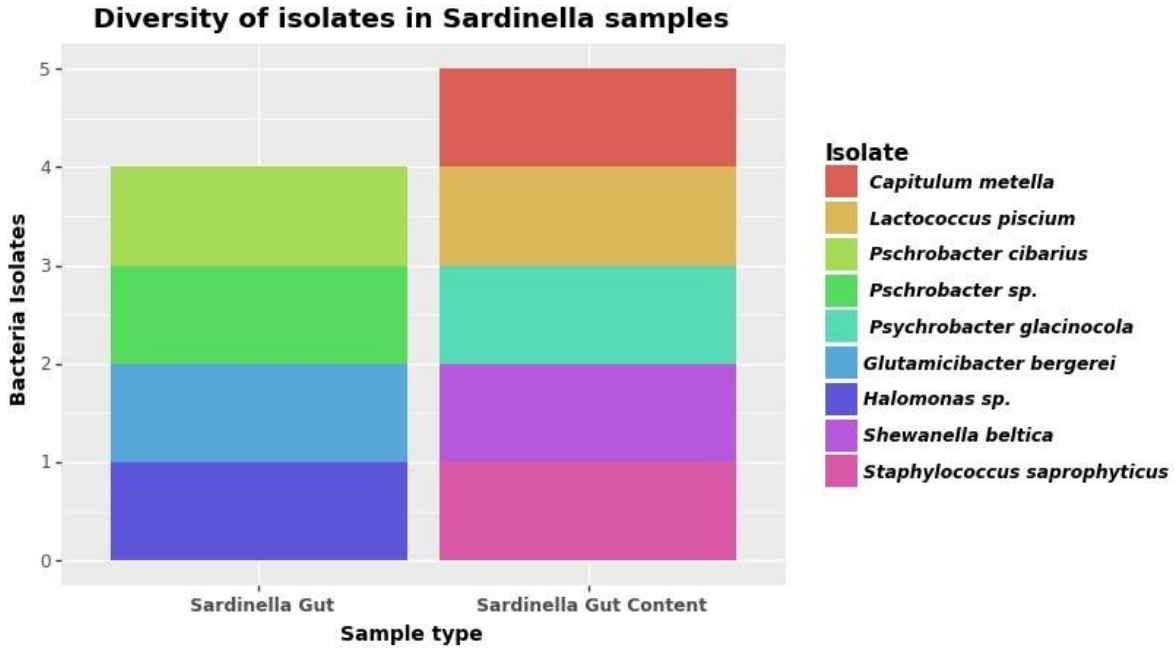


**Figure 9:** Different isolates found in the gut and gut content of sardinella (*S. maderensis*). Each color represents an isolate.

### 4.5. Diversity of bacteria isolates in picarel samples.

Six bacteria isolates were identified and associated with the Picarel (*S. melanurus*) gut and gut content; they belong to four genera. Four different isolates were found in the gut and three in the gut content of picarel, as given in (Figure 10). More bacteria isolate diversity was observed in Sardinella (*S. maderensis*) samples compared to *Picarel* samples.

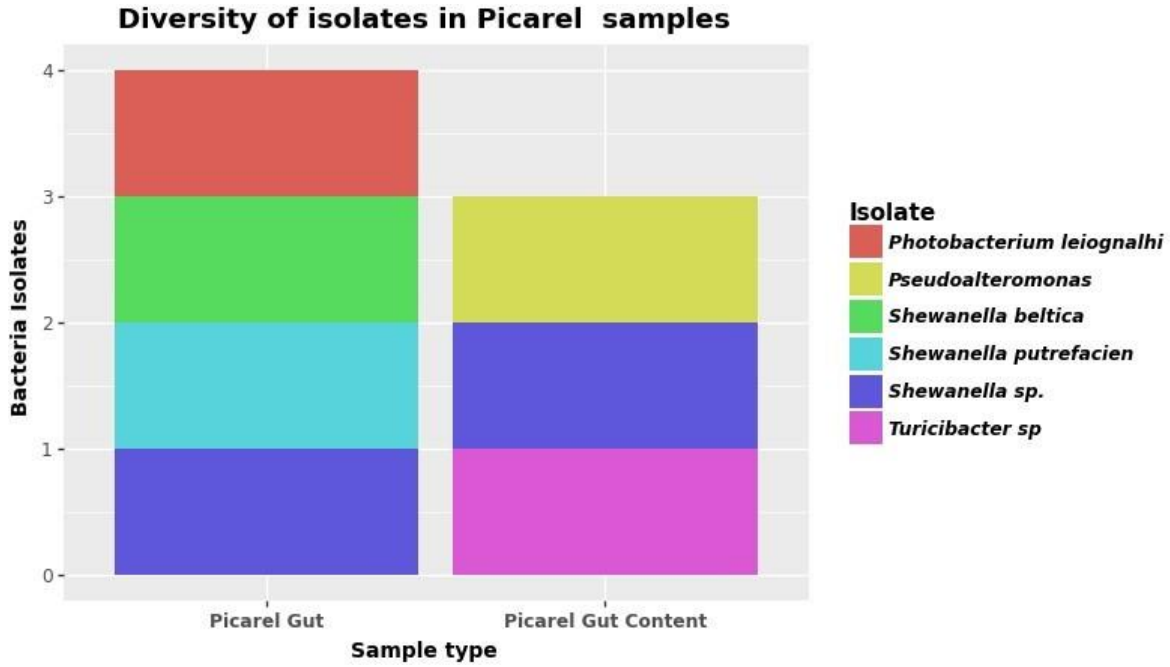


**Figure 10:** Different isolates found in the gut and gut content of picarel *(S. melanurus*). Each color represents an isolate.

### 4.6 Antimicrobial susceptibility analysis

The average diameter of inhibition zone was measured and determined as susceptible, intermediate or resistant. Bacteria with values below the green dotted line were classified as resistant. In between the green and the violet dotted lines were intermediate and above the violet dotted line were considered susceptible. *E. coli* strain was used as control. The susceptibility test revealed a varied degree of resistance to different antibiotics in different samples.

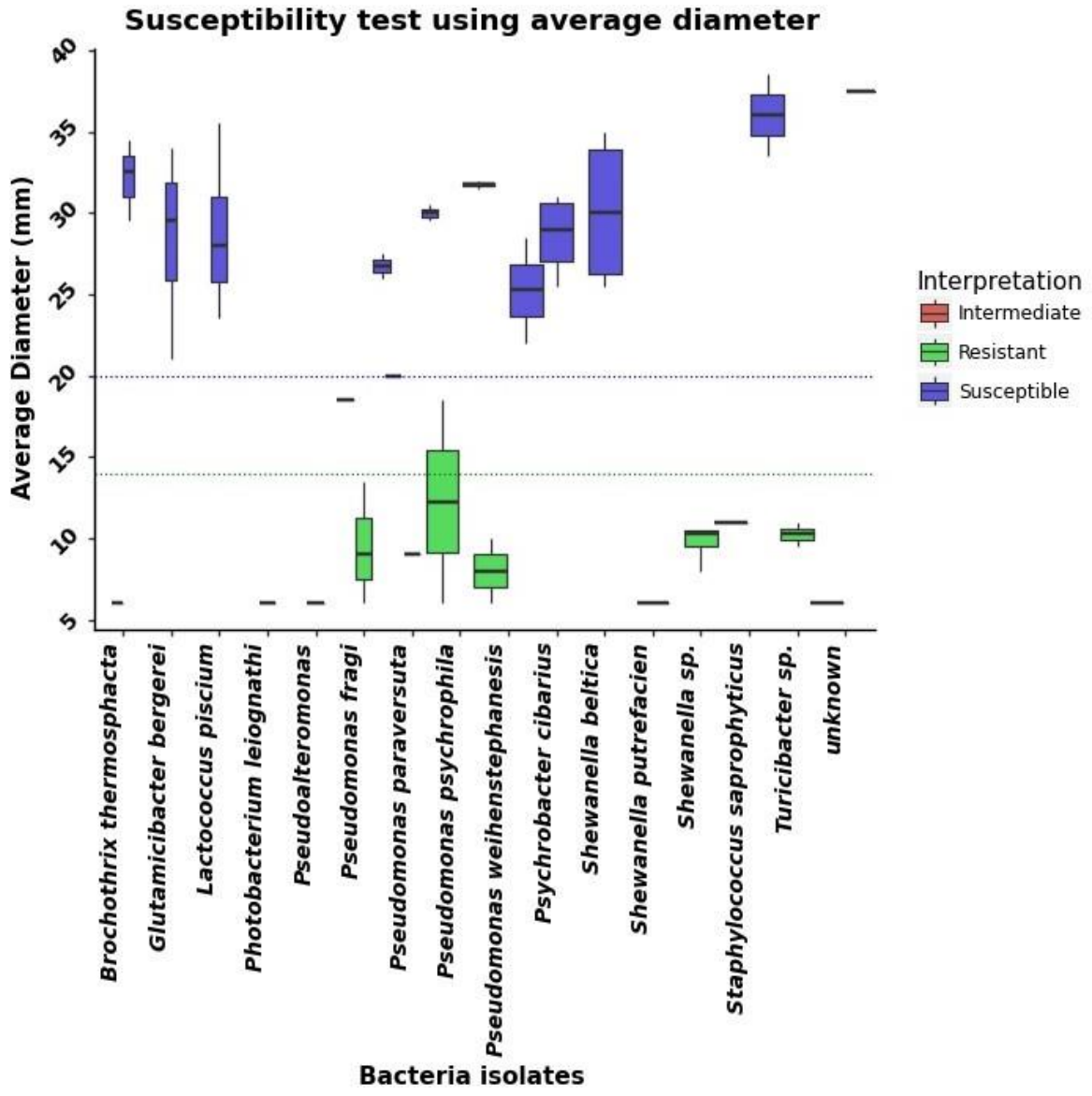


**Figure 11**: Antimicrobial susceptibility analysis using diameter interpretation of inhibition zones of the different antibiotics used in this study.

### 4.7 Antimicrobial susceptibility test in all samples

A total of seventeen bacteria isolates from different samples were tested against four antibiotics, namely ampicillin (10 µg), erythromycin (10 µg), streptomycin (10 µg), and chloramphenicol (30 µg), they can't cross the out membrane of most of the isolates. CLSI enterobacteriaceae breakpoint was used base on genera since the isolate not all were clinical. The identified isolates responded differently to the antibiotics they were tested against, with eight showing multiple antibiotics resistance, five being susceptible to all antibiotics and four being resistant to only one antibiotic, as depicted in (Figure 12).

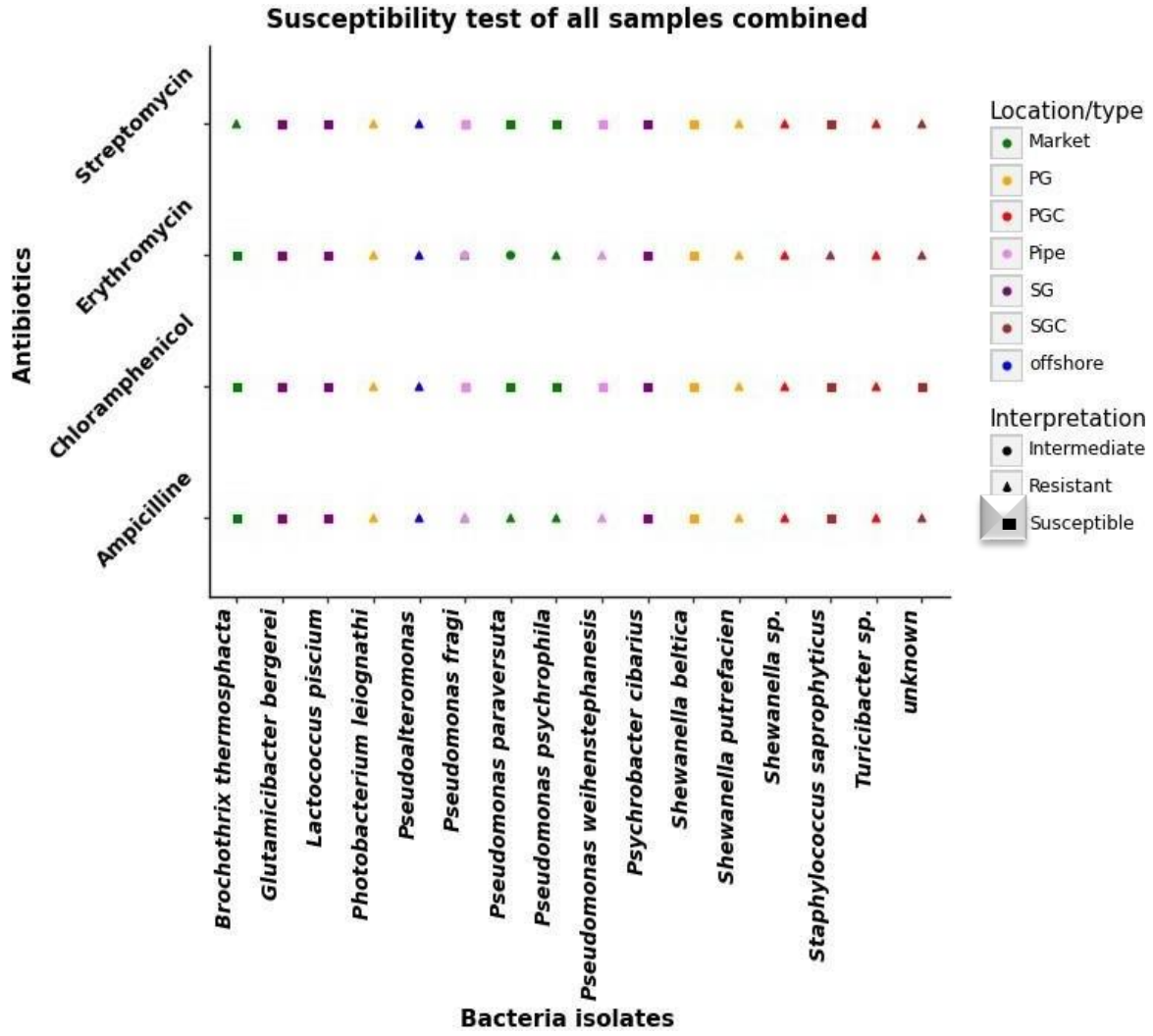


**Figure 12**: Diversity of isolates from different samples tested against four antibiotics. Each colour represents a sample.

### 4.8 Antimicrobial susceptibility test on water samples

Six isolates from water samples were tested and the results showed that five were resistant to ampicillin. *Brothothrix thermosphacta* is the only one that was susceptible and is found in the market samples. All isolates were susceptible to chloramphenicol except *Pseudoalteromonas* which was resistant and found offshore, which might be an indicator of contamination in the area where they are from. A similar occurrence was observed for Streptomycin, but in this case, two isolates were resistant, one from the Market and one from the pipe as shown in (Figure 13).

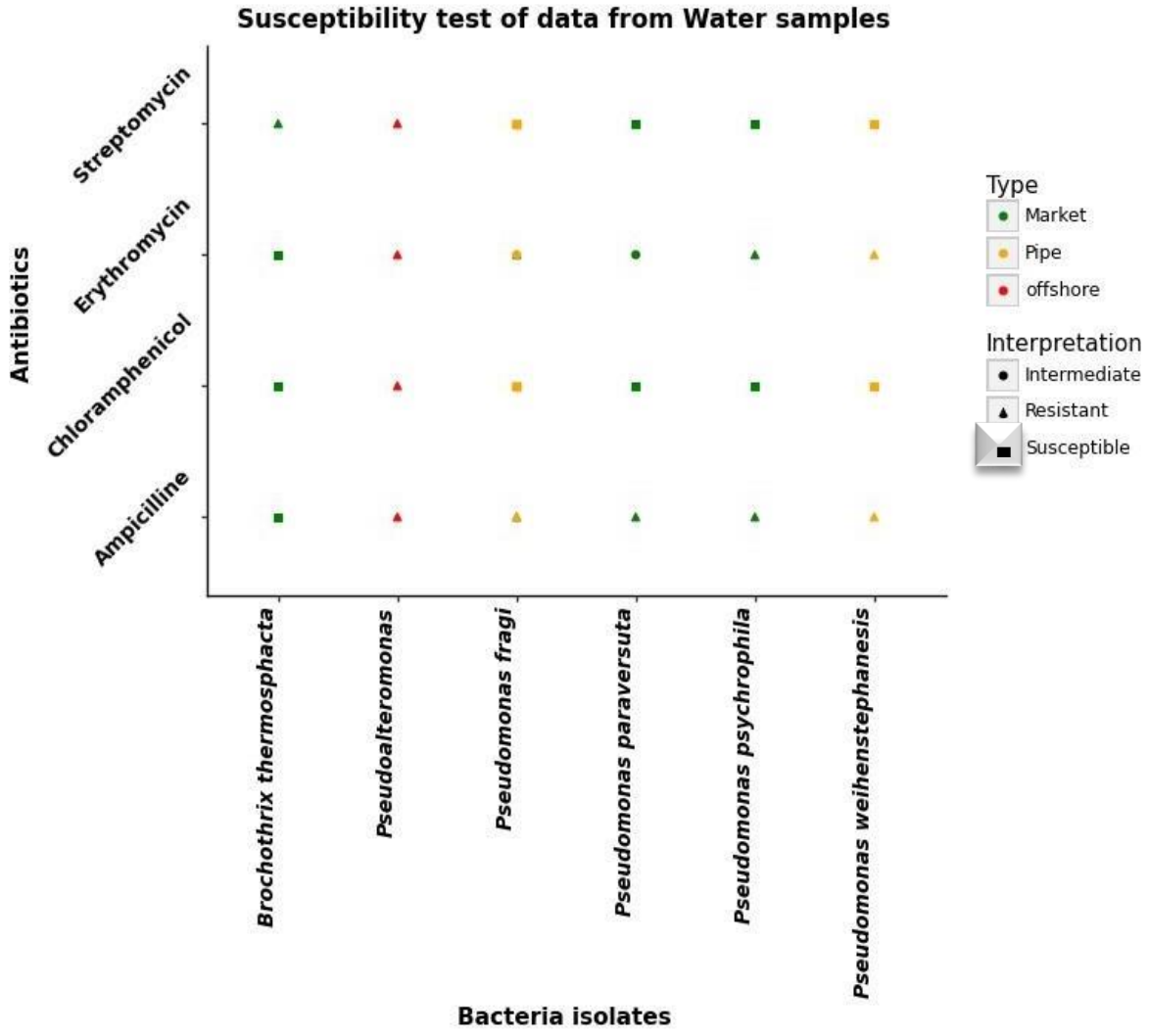


**Figure 13**: Water sample isolates susceptibility test with four antibiotics. Each color represents a location and shape represents the interpretation of susceptibility.

### 4.9 Antimicrobial susceptibility test on sardinella samples

Five isolates from the gut (SG) and gut contents (SGC) of sardinella (*S. maderensis*) were subjected to antibiotic susceptibility testing. All isolates from the gut of sardinella demonstrated susceptibility to the entire panel of tested antibiotics. In contrast, the isolates from gut contents exhibited variability in their antibiotic resistance profiles. One isolate showed susceptibility to all antibiotics except erythromycin, while another showed resistance to all antibiotics except chloramphenicol (Figure 14).

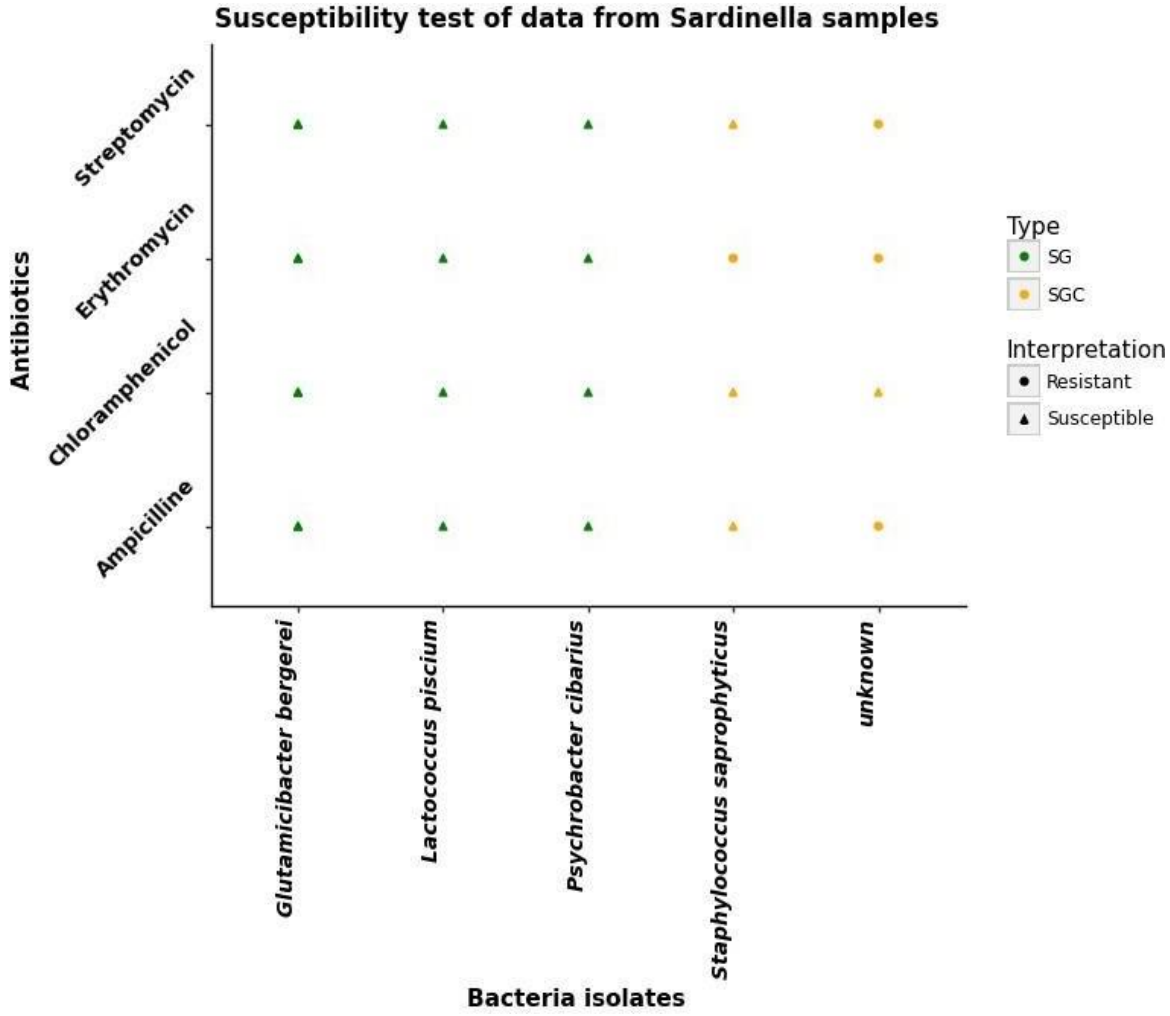


**Figure 14**: Sardinella (*S. maderensis*) isolates susceptibility test with four antibiotics SG is for sardinella gut and SGC is for Sardinella gut content.

### 4.10 Antimicrobial susceptibility test on picarel samples

Five isolates from the gut (PG) and gut content (PGC) of picarel (*S. melanurus)* were subjected to antibiotic susceptibility testing. Two isolates from the picarel gut were resistant to all antibiotics tested and one was susceptible to all antibiotics. Isolates found in the gut content were resistant to all antibiotics, as shown in (Figure 15).

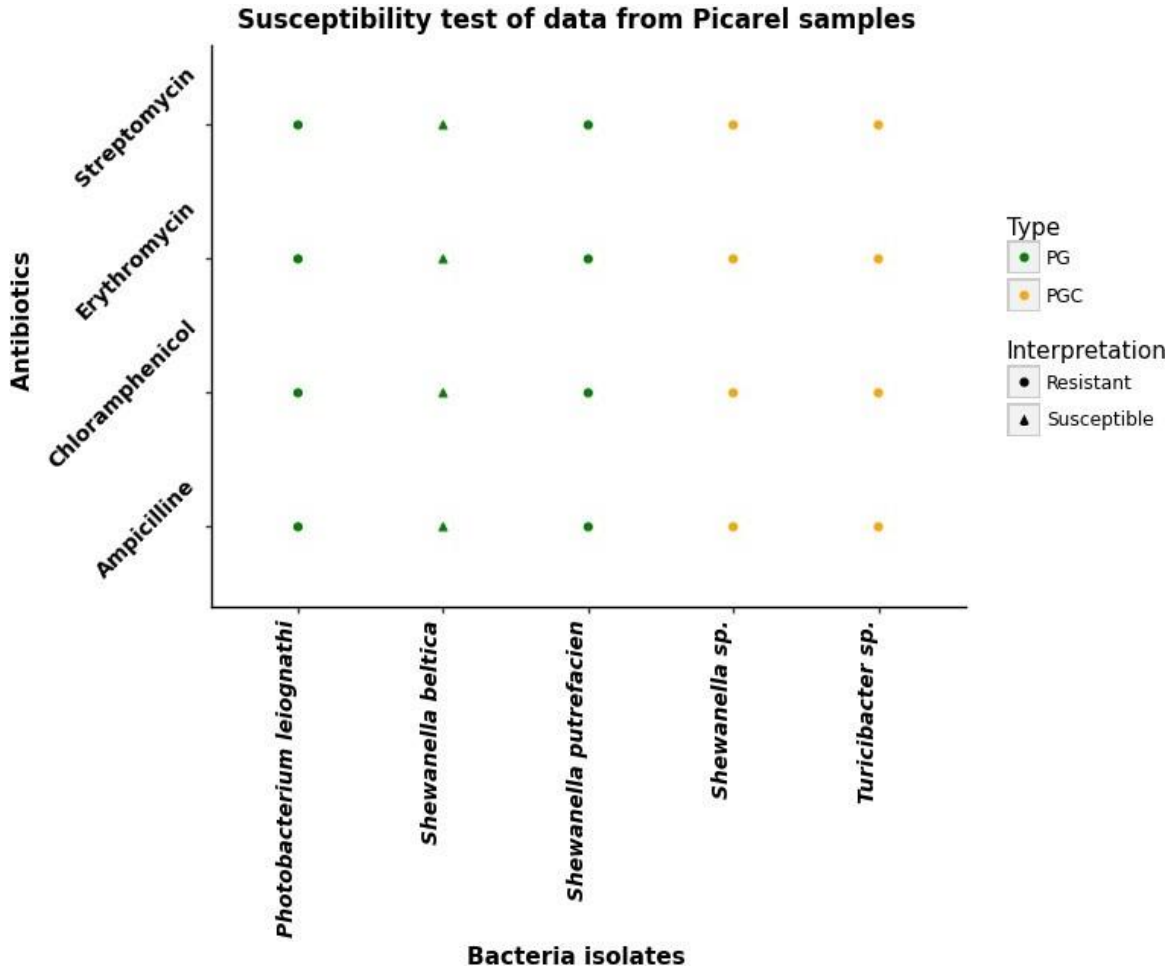


**Figure 15**: Picarel *(S. melanurus*) isolates susceptibility test with four antibiotics PG is for Picarel gut and PGC is for Picarel gut content.

### 4.11 Comparing the antimicrobial susceptibility of the guts and gut contents of the two fish species

The analysis showed higher susceptibility in the sardinella (*S. maderensis*) gut than picarel gut. One picarel (*S. melanurus)* gut isolate showed susceptible to all the tested antibiotic and the other two were resistant to all tested antibiotics. The isolates from the picarel (*S. melanurus)* gut content were resistant to all the antibiotics tested. For the sardinella gut, all isolates were susceptible to all antibiotics tested. For the two isolates tested from sardinella (*S. maderensis*) gut content, one was susceptible to three antibiotics and resistant to erythromycin, while the other showed resistance to all three antibiotics and susceptible to chloramphenicol. The analysis showed a high resistance in the picarel gut content compared to sardinella gut content, see (Figure 16).

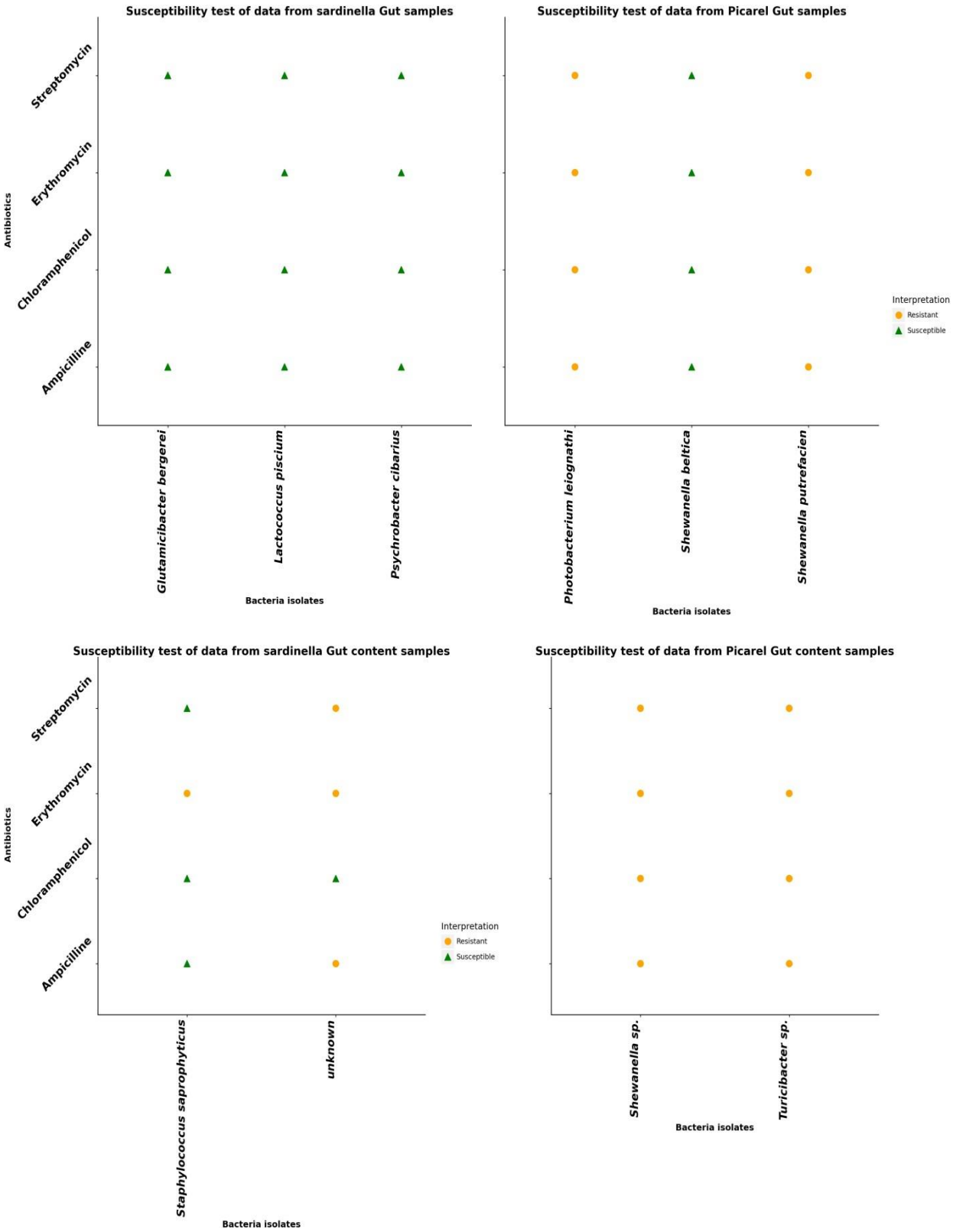


**Figure 16**: Comparing the susceptibility of the guts and gut contents of sardinella (*S. maderensis*) and picarel *(S. melanurus*) isolates.

## 5. Discussion

This study investigated bacterial load and antibiotic resistance in fish and water samples from the Mindelo Fish Market in São Vicente, Cabo Verde. It was found that picarel (*S. melanurus*) has a higher cfu/g count than sardinella (*S. maderensis*). This could be because of their feeding habit and host phylogeny. Picarel is carnivorous as it feeds on small vertebrates and phytoplankton while sardinella is herbivorous, feeding mainly on phytoplankton. Diversity in their feeding habits, and their phylogenetic relationships play a significant role in shaping their dietary preferences and ecological roles within aquatic ecosystems (Sullam et al., 2012). The water samples also showed the pipe has a higher cfu/ml count than the waters adjacent to the fish market. This can be explained by the fact that the fish cleaning waste at the time of the collection was highly concentrated in the pipe before entering the water body. Fish cleaning waste can introduce contaminants and pathogens into the water. These contaminants may include heavy metals, oils, and chemicals used during fish cleaning (Byrd et al., 2021). Pathogens from fish waste can contaminate the water, making it unsafe for swimming, fishing, or other recreational activities (Some et al., 2021). Additionally, the presence of pathogens can lead to disease outbreaks in fish and other aquatic organisms.

Water is one of the most important bacterial habitats on Earth. As such, water also represents a major way of disseminating bacteria between different environmental compartments. Human activities led to the creation of the so-called urban water cycle, comprising different sectors (waste, surface, drinking water), among which bacteria can hypothetically be exchanged. Therefore, bacteria can be mobilized between unclean water habitats (e.g. wastewater) and clean or pristine water environments (e.g. disinfected and spring drinking water) and eventually reach humans. In addition, bacteria can also transfer mobile genetic elements between different water types, other environments (e.g. soil) and humans. These processes may involve antibiotic resistant bacteria and antibi- otic resistance genes. Antibiotics are commonly used to prevent and control diseases in aquaculture. However, long-term/overuse of antibiotics not only leaves residues but results in the development of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs).

The study of antibiotic resistance in bacteria from fish is important because it may indicate the extent to which anthropogenic activities have altered water ecosystems. Aquatic bacteria can be either indigenous to aquatic environments or exogenous, meaning they are present in the water only briefly and infrequently because of shedding from animal, vegetal, or soil surfaces. The antibiotic resistance of the strains could be explained by the possibility of heavy use of

antibiotics like chloramphenicol in aquaculture, several of which are non-biodegradable, increasing antibiotic selective pressure in water and facilitating the transfer of antibiotic-resistant determinants between aquatic bacteria, including fish and human pathogens, and allowing the presence of residual antibiotics in commercialized fish and shellfish products (Alanis, 2005; Alonso et al., 2001).

A high population of bacteria association with the fish is a combination of the natural state that the samples would have in the natural environment plus any potential growth of bacteria after its catch and storage at the fish market. Ampicillin resistance was notable (Thomassen et al., 2022; Yousfi et al., 2017) with *Pseudomonas* and *Shewanella*. These results match many studies that found *Shewanella putrefaciens* isolated from shellfish collected from the West Sea in Korea showing resistance to multiple antibiotics, including ampicillin, cefotaxime, and tetracycline (Yousfi et al., 2017; Kang et al., 2013). Ben Mhenni et al., (2023), isolated *Pseudomonas spp*. from fresh fish fillets and identified as being resistant to at least one antimicrobial, mainly ampicillin, amoxicillin, and tetracycline. A large proportion of *Pseudomonas* isolates associated with salmon processing environments were resistant to ampicillin and amoxicillin (Ben Mhenni et al., 2023; Thomassen et al., 2022). Ampicillin resistance is a major concern, as seen by the variable, vast range of dimensions and physical measurements on the samples. The resistant, intermediate, and susceptible groups had statistically significant diameter variations. Developing alternative methods for controlling bacterial contamination in aquatic environment and fish processing. This could include the use of non-antibiotic antimicrobial agents or novel preservation techniques

This study confirms previous findings on aquatic environment bacterium diversity and antibiotic resistance (Vaz-Moreira et al., 2014; Alonso et al., 2001). *Glutamicibacter Bergerei, Pschrobacter, Staphylococcus Saprophyticus, and Shewanella* have been found in aquatic settings and aquaculture systems (Odeyemi et al., 2020). Antibiotic resistance, particularly to ampicillin, matches global patterns in aquatic environments (Larsson et al., 2018). “Genetic mutations, horizontal gene transfer, and antibiotic genes have been studied in several bacterial species” (Castillo et al., 2015). Understanding these pathways is crucial to developing antibiotic resistance-fighting tactics. This result supports prior research that emphasizes surveillance, appropriate antibiotic use, and alternative treatment ways to prevent antibiotic resistance (Stalder et al., 2012).

The susceptibility test provided important insights into the resistance patterns of the bacterial isolates against commonly used antibiotics. The disc diffusion method revealed the zones of

inhibition, indicating the effectiveness of antibiotics against the tested isolates. Notably, the presence of resistance to ampicillin among the isolated raises concerns about the spread of antibiotic resistance. The high prevalence of ampicillin resistance observed in this study is consistent with global antibiotic resistance trends in aquatic environments (Schar et al., 2021). The emergence of antibiotic resistance can be ascribed to a multitude of factors, encompassing the extensive utilization of antibiotics in aquaculture, environmental contamination, and the horizontal transfer of resistance genes (Levy, 2002). Additional research is imperative to unravel the fundamental mechanisms responsible for ampicillin resistance within the examined isolates. Understanding the behaviors of antibiotic resistance is crucial for developing effective strategies to combat bacterial infections.

The finding of this research has significant environmental and public health implications. Bacteria may have ecological roles in aquatic environments due to their abundance in samples. *Pseudomonas* and *Shewanella,* two of the species found in this study, may cause food spoilage and are a risk to human health if they proliferate excessively in fish. Understanding these species' abundance and range may aid food production and aquatic habitat risk assessment and management. Antibiotic usefulness in treating bacterial infections is questioned by antibiotic-resistant bacterial isolates. The findings emphasize the need for antibiotic resistance monitoring and alternative therapy development. The development of high ampicillin resistance also stresses the need for antibiotic stewardship in aquaculture and medicine.

## 6. Conclusions

This research highlights aquatic bacterial species composition, antibiotic sensitivity, and resistance trends. Identification of *Photobacterium leiognathi*, *Turicibacter*, *Shewanella putrefacien*, *Pseudoalteromonas*, and *Brochothrix* in this study demonstrate the complexity of fish and water microbial communities. They are known to be specific spoilage organisms (SSO). Seawater can serve as a source of initial contamination and eventual spoilage of fish in addition to the normal flora present on the fish and in the gastrointestinal tract at the time of harvest. These isolates showed resistance to all antibiotics.

This research impacts environmental and public health. Antibiotic-resistant microorganisms cast doubt on antibiotic efficacy. Ampicillin resistance was widespread, highlighting the need for antibiotic prudence and new treatments. The discovery of numerous bacterial species, some of which cause food deterioration and human diseases, emphasizes the need for risk assessment and management in food production and aquatic environments. Antibiotic resistance requires interdisciplinary approaches. Aquatic antibiotic resistance monitoring programs should be robust to detect resistance patterns, monitor resistant strains, and guide effective intervention options. Aquaculture and healthcare settings must also utilize antibiotics responsibly to reduce antibiotic resistance.

This research sheds light on bacterial load and antibiotic resistance in fish and water. The study had some area of limitations that could be improvement in future study in this sector. The small sample size limits the investigation, the research used a limited number of samples from fish species, which may not effectively reflect bacterial load variety and variability. Understanding bacterial load and antibiotic resistance trends requires a bigger and more varied sample. The disc diffusion process is another limitation of antibiotic susceptibility testing. This approach is frequently used and gives significant information, but it has disadvantages including varying interpretation criteria and false-positive or false-negative outcomes. Future antibiotic susceptibility testing may include both microdilution or molecular methods to enhance accuracy and reliability. Future studies should use molecular methods like whole-genome sequencing and gene expression analysis to understand aquatic antibiotic resistance.

Additionally, the study's single location and temporal scope may restrict the wide applicability of its findings. To further understand bacterial diversity and antibiotic resistance trends, future research should include more places and last longer to capture seasonal and temporal fluctuations.

## 7. Recommendations

The current study's results and limitations suggest many directions for future research on bacterial species distribution and antibiotic resistance in aquatic settings.

- Firstly, bigger and more representative sample sizes are needed to capture true bacterial diversity and heterogeneity across geographic areas and aquatic systems. A larger sample size will help determine bacterial species predominance and antibiotic resistance tendencies. This applies to both the number of fish species as well as the number of bacteria selected. It is important to note that our diversity analysis was intentionally picking morphological differences in the bacterial colonies so should characterize the most obvious different but within similar morphologies there may be differences, additionally, by picking more colonies it would be able to more accurately determine the community structure of the cultivable bacteria.
- Secondly, complementary and alternative methods may increase antibiotic susceptibility testing accuracy and reliability. Broth microdilution may help to isolate different bacteria while molecular methods like PCR and DNA sequencing may reveal more about the diversity of uncultivable bacteria.
- Thirdly, it would be important to try to isolate bacteria on freshly collected samples as freezing (at -80 $^{o}$C) and then thawing may influence the overall CFU or diversity collected.
- Finally, research should focus on antibiotic resistance understanding and prevention. This involves encouraging ethical antibiotic use in aquaculture, tracking antibiotic resistance trends, and exploring antibiotic-free therapeutic alternatives. Future research can help develop evidence-based antibiotic resistance management strategies, understand bacterial species distribution and antibiotic resistance in aquatic environments, and protect human and environmental health by implementing these recommendations.

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# 9. Appendix

## 9.1 Inoculation Protocol

1. Prepare the Culture Medium:

a. Select an appropriate culture medium based on the type of organism you are working with (agar plates).

MA 400ml: 14.96g MA +6g of Agar diluted with 400 ml of milli cube water.

NA 400ml:1.2g of yeast +2g peptone+3.2g of sodium chloride Nacl+6g agar with 400ml millicube water

Peptone water 0.1%: 0.4g of peptone diluted with 400 ml milli cube water.

2. Sterilize Equipment and Workspace: a. Ensure that all equipment, including inoculating loops or needles, pipettes, and culture vessels, are properly sterilized using autoclaving or other suitable sterilization methods.

b. Clean the workspace with a suitable disinfectant to maintain a sterile environment.

3. Prepare the Inoculum:

a. Pour already prepared media into sterilized plates and leave to solidify.
b. Prepare serial dilutions $10^{-1}$-$10^{3}$ for the samples:9 ml Peptone/PBS+1 ml from the sample.

4. Inoculation: a. For Agar Plates:

a. Flame-sterilize an inoculating loop or needle until it is red-hot.
b. Allow the loop/needle to cool by touching it to the sterile surface of the agar away from the desired inoculation site.
c. Lift the lid of the agar plate just enough to access the surface without contaminating it. Avoid prolonged exposure of the agar to the air.
d. In a smooth and continuous motion, streak the loop/needle/beat over the agar surface, depositing the inoculum. Typically, streaking can be done in a zigzag or quadrant pattern. v. Close the lid of the agar plate immediately after streaking and secure it with tape or parafilm. vi. Sterilize the loop/needle again by flaming it before proceeding to the next plate.

4. Incubation:

a. Place the inoculated culture plates or tubes in a suitable incubator set at the optimal temperature and atmospheric conditions for the growth of the target organism.

b. Follow the recommended incubation time specific to your organism or experimental requirements.

Remember to maintain proper sterile techniques throughout the process to avoid contamination and ensure accurate results. Adapt the protocol as necessary based on the specific requirements of your experiment or the organism being cultured.

**9.2 PCR Protocol**

1. Sample Preparation:

a. Prepare the DNA sample and properly label and store it in a -20 freezer.

b. Thaw the DNA sample on ice if you are about to start work.

2. PCR Reaction Setup:

a. Prepare a PCR reaction mix containing the following components:

DNA template (target DNA): 1

Forward and reverse primers (specific to the target DNA): 27F 10 um 0.8 and 1392-R 10 um 0.8

dNTPs (deoxynucleotide triphosphates): 10 um 0.4

PCR buffer (containing necessary salts and buffer components): 2.

DNA polymerase (Taq polymerase is commonly used): 0.5.

Molecular-graded water: 14.5

b. Calculate the volumes of each component based on the number of reactions and their concentrations. Prepare a master mix with enough volume for all reactions plus a small extra amount to account for pipetting errors.

c. Add the appropriate volumes of each component to a sterile PCR tube or plate. Mix the reaction mix gently.

PCR Cycling: The PCR reaction typically involves repeated cycling through three different temperature steps: Denaturation, Annealing, and Extension.

a. Denaturation:

Initial denaturation: Heat the reaction mix to 95°C for 5 minutes to denature the DNA strands.

b. Cycling:

Denaturation: Heat the reaction mix to 95°C for 30 seconds to separate the DNA strands.

Annealing: Cool the reaction mix to a temperature specific to the primers used. The annealing temperature depends on the primer sequences and is typically 53°C. Annealing occurs for 30 seconds.

Extension: Raise the temperature to 72°C (or according to the optimal temperature for the DNA polymerase) for a sufficient duration to allow DNA polymerase to extend the primers and synthesize new DNA strands. Extension times vary based on the length of the target DNA but usually for 2 minutes per kilobase.

c. Final Extension:

After the final PCR cycle, perform a final extension step at 72°C for 10 minutes to ensure complete extension of any remaining incomplete DNA strands.

PCR Amplification Cycles: The number of cycles depends on the starting concentration of the target DNA and the desired level of amplification. Typically, 35 cycles are performed.

Final Hold: After the cycling is complete, hold the reaction mix at 4-10°C to maintain stability or transfer it to a freezer for long-term storage.

It's important to note that specific parameters such as primer design, cycling conditions, and reaction volumes may vary depending on the specific application and target DNA. Always refer to the manufacturer's instructions for the PCR reagents and equipment you are using for more detailed protocols.

**9.3 Gel Run Protocol**

1. Prepare the Gel:

a. Determine the appropriate type and percentage of gel based on the size range of the molecules you are separating. Commonly used gels include agarose.

b. Prepare the gel according to the manufacturer's instructions or follow a standard gel recipe. 2.25 of agarose in a suitable buffer (TAE 150ml for agarose gels) and heat it until fully dissolved in a microwave.

c. Allow the gel mixture to cool to a temperature where it can be handled safely, typically around 50-60°C.

2. Add Gel Components:

a. Using agarose gel, add a suitable DNA stain (ethidium bromide six drops) to the gel mixture while it is still liquid and mix well.

b. Pour the gel mixture into a gel tray or gel-casting apparatus, ensuring that it is level and free of bubbles. Insert a suitable comb at one end of the gel to create wells for sample loading.

c. Allow the gel to solidify completely, typically for 15 minutes.

3. Prepare the Running Buffer: a. Determine the appropriate buffer system based on the type of gel and the molecules being separated. Commonly used buffers include TAE (Tris-acetate-EDTA).

b. Prepare a sufficient volume of the running buffer according to the manufacturer's instructions or using a standard recipe. Ensure that the buffer is fully dissolved and at the appropriate ph.

4. Sample Preparation:

a. Prepare your samples by mixing them with an appropriate loading buffer (loading dye). The loading buffer aids in sample loading and provides density for sample tracking during electrophoresis.

b. Heat the sample and load buffer mixture, if necessary (e.g., when using DNA samples), to denature the molecules and ensure uniform migration during electrophoresis.

c. Briefly centrifuge the samples to collect any liquid at the bottom of the tubes.

5. Load the Gel:

a. Carefully remove the comb from the gel, leaving wells for sample loading.

b. Using a micropipette, load the samples into the wells, ensuring accurate pipetting and avoiding cross-contamination between samples: Sample 5 ul +1 ul dye.

c. ladder created in the first wells to serve as a reference for determining the size of the separated molecules: DNA ladder 1 ul+1 ul dye+8 ul $H_2O$

6. Run the Gel:

a. Place the gel in the electrophoresis apparatus, ensuring that the gel is fully immersed in the running buffer.

b. Connect the leads from the power supply to the electrodes of the apparatus, ensuring correct polarity (positive to the anode and negative to the cathode).

c. Set the desired voltage or current based on the gel type and expected separation requirements. A typical voltage of 100 volts is commonly used for agarose gels.

d. Run the gel for the desired duration, typically 30 minutes to a few hours, depending on the size of the separated molecules and the gel type.

7. Visualize the Results:

a. After electrophoresis, carefully remove the gel from the apparatus and place it on a gel tray or transilluminator.

b. Visualize the separated molecules using an appropriate detection method, such as UV light for gels stained with ethidium bromide.

c. Document and analyze the results, noting the migration distances and band patterns.

Remember to follow appropriate safety measures and manufacturer's instructions throughout the procedure, including handling of DNA stains, disposal of gel waste, and proper handling of electrical equipment. Adjustments to the protocol may be necessary based on specific gel types, equipment, or applications.

**9.4 Prepare Sample for Sanger Sequencing Protocol**

a. Stick barcodes to 1.5-mL centrifuge tubes. The barcodes are found in the plastic box at the back of the lab on the east shore.
b. Write down the barcode and corresponding isolate name on your lab notebook.
c. Transfer the PCR products into the appropriate tube.
d. Add 5 μL of 10 μM 27-F primer to each tube.
e. Place tubes in a sealed envelope or Ziploc bag.
f. Enter sequencing order in David’s Eurofins account following his instructions or ask him to help you do it.

## Data availability

I collected the data myself it can be accessible upon request through my email: andure@uta.cv through approval from WASCAL Cabo Verde and will also archive in the WASCAL website too: www.wascal.org.